(54) Title: SUPERCRITICAL FLUIDS PROCESSING: PREPARATION OF PROTEIN MICROPARTICLES AND THEIR STABILISATION

(57) Abstract: A process for the co-precipitation of a substance with a stabiliser therefor, by a gas anti solvent process comprising introducing into a particle formation vessel a supercritical fluid pure or mixed with a modifier, and a solution comprising said substance and said stabiliser dissolved in a solvent; so as said solvent is extracted from the solution by said supercritical fluid and co-precipitation of the substance and stabiliser occurs. The process may be carried out using an apparatus comprising a particle formation vessel (22) and a nozzle (27) having a central orifice (39) serving to introduce a solution of the substance and a plurality of outer orifices (41) serving to carry a flow of supercritical fluid into the particle formation vessel (22), such that the solvent is extracted from the solution by the supercritical fluid and precipitation of micron sized particles of the substance/stabilizer occurs. The substance is preferably a protein of pharmaceutical interest, which is highly stabilized for storage by this process.

(15) Information about Correction:
see PCT Gazette No. 46/2003 of 13 November 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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SUPERCRITICAL FLUIDS PROCESSING: PREPARATION OF PROTEIN MICROPARTICLES AND THEIR STABILISATION

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Field of invention

The present invention relates to a method for protein and polypeptide precipitation by supercritical fluid processing and their protection and stabilisation

10   against denaturation.

Background of the invention

The need for stable proteins and polypeptides for many applications is continuously increasing. This is

15   particularly pronounced for therapeutic proteins in the pharmaceutical field. For the ease of both manufacturers and final users aqueous protein solutions are often the preferred form of administration. Moreover this is their common natural form that allows

20   hydrated, three-dimensional folded complex formation. This conformation is generally reported as tertiary structure and its integrity is of vital importance for maintaining the biological activity of proteins. The irreversible loss of tertiary structure of proteins is

25   referred as denaturation and causes inactivation. Because proteins and polypeptides in solution are exposed to many stresses which can cause physical (denaturation) and chemical (i.e. reactions such as hydrolysis, deamidation etc...) degradation, very often

30   the development of liquid formulations is precluded. Presently, the most common way to achieve protein stability is the removal of water by suitable processes such as freeze-drying or spray-drying. However, both of these techniques *(ref. "Formulation and Delivery of*

35   *Proteins and Peptides" J. L. Cleland and R. Lan ger American Chemical Society, Washington, DC 1994)* can induce protein unfolding. In particular with regard to

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lyophilisation protein unfolding can occur either during
the initial freezing step or during acute dehydration by
sublimation.

Concerning spray drying, thermal degradation, low
5   efficiency, low yield and high levels of residual
moisture are the main limitations of the technique.

Another problem is the difference in long term
stability of analogous formulations obtained by
different drying processes. In fact, depending on the
10   dehydration method, the protein may assume different
three-dimensional structures with the same initial
biological activity but different shelf life.

The stabilising effect of carbohydrates and in
particular of trehalose on proteins during freezing and
15   dehydration is well documented ("Formulation and
Delivery of Proteins and Peptides" J.L. Cleland and R.
Lan ger American Chemical Society, Washington, DC 1994
and "Freeze-Drying/Lyophilization of Pharmaceutical and
Biological Products" L. Rey and J.C.May, Marcel Dekker,
20   Inc. New York 1999). Although many sugars can prevent
protein damage during dehydration, the products often
have a short shelf life at room temperature due to the
Maillard reaction. Stability at room temperature can be
improved using non reducing sugars such as sucrose and
25   trehalose.

British Patent Application GB 2009198 discloses
lyophilisation of meningococcal polysaccharide and
trehalose; GB 2126588 discloses the stabilisation of
tumour necrosis factor (TNF) to lyophilisation and
30   freezing by including either a non-ionic surfactant or
trehalose (or another sugar); and Japanese Patent
Application J 58074696 discloses the freeze-drying of
ATP in presence of trehalose.

Preparations of alkaline phosphatase containing
35   trehalose are reported to maintain their activity after
freeze-drying and to maintain about 70% of the initial
activity after 84 days/storage at 45°C (A.W. Ford et

Al., J.Pharm.Pharmacol. 1993, 45: 86-93). Although
lyophilisation is still the main process used for drying
proteins, several precautions must be taken in order to
avoid the damage that severe stressing phases such as
freeze-thawing and drying can cause. In fact, during
the first step in freeze-dried protein formulation, a
correct choice of conditions (pH, ionic strength,
presence of stabilisers, etc...) guarantees the best
protection against protein unfolding and inactivation.
Many excipients such as sugars, aminoacids, polymers,
surfactants specific ligands (substrates, co-factors,
allosteric modifiers etc...) are known to stabilise
proteins during freeze-drying and have been named
"lyoprotectants". Among them, carbohydrates and in
particular disaccharides such as sucrose and trehalose
have been widely studied. The stabilising mechanism of
these compounds as well as other stabilisers has not
been completely clarified. However, an effective
lyoprotectant must maintain stability during both
freeze-thawing and drying. Since the protein
environment is aqueous during much of the freezing
process, solutes that stabilise the native conformation
in aqueous solutions are very often effective as protein
cryoprotectants. Carbohydrates and some aminoacids are
examples. Arakawa et Al. (J.Pharm.Res. 1991, 8, 285-29
1) reported that such solutes tend to be excluded from
the surface of protein while in aqueous solution. The
thermodynamic consequence of such phenomenon is the
stabilisation of protein native conformation.

Stability during drying and storage is best
explained by both the water substitution and the
vitrification hypotheses. The first states that
stabilisers interact with the protein as water does by
replacing the removed water and accounts for the
thermodynamic control of drying process. The latter
states that stabilisers are good glass formers and
remain amorphous during and after drying so that they

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mechanically immobilise proteins inside a glassy matrix.
This is a purely kinetic argument that applies equally
well to both drying and storage stability. ("Freeze-
Drying/Lyophilization of Pharmaceutical and Biological

5   Products" L. Rey and J. C. May, Marcel Dekker, Inc. New
York 1999).

Hence, referring to the above stabilisation
hypothesis of dried proteins, it can be postulated that
vitrification is one of the main issues for long term

10   stability. The use of spray-drying for protein
desiccation has been less investigated. Although fine
amorphous particles can be produced, this process
requires warm air as a drying force that can lead to
protein thermal degradation. Moreover, low efficiency,

15   low yield and high levels of residual moisture are other
limitations.

Another reported technique for drying proteins
which should avoid inactivation is air dehydration at
room temperature. US4,891,319 of Quadrant Bioresources

20   Ltd (UK) discloses the preservation of several proteins
and other macromolecules at 37-40°C by drying in
presence of trehalose at atmospheric pressure.

The use of supercritical fluid technology has also
been reported as a useful method for obtaining proteins

25   as dry fine micro-particles. The main advantages of
this technique are the possibility of maintaining the
protein in a favourable aqueous environment before a
rapid precipitation in order to minimise denaturation
and the process length which is shorter than freeze-

30   drying and less expensive.

S.P. Sellers et Al. (J. Pharm. Sci., 2001, 90, 785-
797) report a dehydration method for protein powder
production based on supercritical CO_2-assisted
nebulization. This technique can be assimilated to

35   spray-drying; in fact supercritical CO_2 is used for
enhancing solution nebulization and not as an anti-
solvent for solute precipitation. The GAS (Gas Anti-

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Solvent recrystallization) process to form protein
microparticles is reported by Debenedetti *(US
6,063,910)*. In this case the protein solution is
sprayed through a laser drilled platinum disc with a
5   diameter of 20 μm and a length of 240 μm inside the
particle formation vessel previously filled by
supercritical fluid which is introduced by a different
inlet. This technique has been used to form particles
of catalase and insulin (0.01% w/v) from ethanol/water
10   (9:1 v/v) solutions using carbon dioxide as the
supercritical fluid. In this process, the supercritical
fluid inlet is not optimized: the solution injection
occurs in an almost static atmosphere of supercritical
fluid, with low turbulence. Hanna M. and York P.
15   *(WO96/00610)* proposed a new method and a new apparatus
to obtain very small particles by a specific
supercritical fluid technique named SEDS (Solution
Enhanced Dispersion by Supercritical Solution).
         The process is based on a new coaxial nozzle: the
20   solution expands through a capillary inlet,
supercritical fluid expands through an external coaxial
pathway with a conical shaped end. The mixing between
supercritical fluid and solution occurs in the conical
zone. They also propose the use of a three way nozzle:
25   a modifier can be fed in order to improve the mixing.
They applied the SEDS technology to precipitation of
small particles of water soluble compounds, such as
sugars (Lactose, Maltose, Trehalose and Sucrose) and
proteins (R-TEM beta-lactamase). Co-precipitation of
30   proteins and stabilisers is not mentioned nor
exemplified therein.
         Moreover, the same inventors *(WO01/03821)* describe
an improved precipitation method using the same
apparatus but feeding to the particle formation vessel a
35   supercritical fluid and two immiscible solvents. This
.method allows co-precipitation of two or more solutes
dissolved in the two immiscible solvents. The fluids

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inlet is formed by a coaxial nozzle wherein contact
between the two solvents occurs shortly before their
dispersion by the supercritical anti-solvent, avoiding
the precipitation solutes inside the nozzle. However
5   this method permits the formation of homogeneous co-
precipitates; it is generally useful when two solutes
with different polarity must be processed. Moreover, if
this is used for an aqueous solution, the second solvent
must be at least partly soluble in water so that it
10   allows the water to disperse in the supercritical anti-
solvent. This step is necessary to permit water-soluble
solute precipitation. Co-precipitation of proteins and
stabilisers is not described in this document.
          Walker (WO01/15664) discloses a method for co-
15   formulating an active (preferably a pharmaceutically
active) substance and an oligomeric or polymeric
excipient in which an amount between 80 and 100% of the
active substance is in amorphous as opposed to
crystalline form. In these formulations the active
20   substances are more stable compared to the crystalline
forms when stored at temperature between 0 and 10°C.
Only the co-formulation of a pharmaceutical active
substance with an oligomeric or polymeric excipient is
disclosed and there is no mention of protein
25   stabilisation in this document. Protein stabilisation
is therefore achieved in the art through freeze-drying
and spray-drying. The co-precipitation of proteins with
stabilisers using supercritical fluids has not been
described before and it is the object of the present
30   invention.
          We have now found a method of producing stable dry
protein microparticles by co-precipitation with a
stabiliser using supercritical fluids. Preferred
stabilisers are carbohydrates, aminoacids, surfactants
35   and polymers. More preferably the stabilizer is a
sugar, most preferably trehalose.
          Co-precipitation allows intimate interactions

between the protein/stabiliser molecules and an optimal
weight/weight ratio exists for each couple
protein/stabiliser.

In fact since there is no freeze-thawing there is
no need for cryoprotection. Moreover, although the
nature of protein/stabiliser interactions has to be
better clarified, in the present case the stabiliser
plays the essential role of improving storage stability
rather than that of retaining protein activity during
drying. In fact, precipitation by a supercritical fluid
allows by itself protein particle production without
denaturation during the drying process.

Statement of the invention

The term "supercritical fluid" means a fluid at or
above its critical pressure and its critical
temperature.

The term "solvent" means a liquid, which is able to
form a solution with the protein and the stabiliser.

The term "stabiliser" means a solid pharmaceutical
excipient which is able to stabilise, for example,
proteins, which is soluble in the solvent and which is
substantially insoluble in the supercritical fluid.

The term "modifier" is a substance, preferably a
solvent, which enhances the solubility of the "solvent"
in the supercritical fluid.

The present invention provides a process for the
co-precipitation of a substance with a stabiliser
therefor, by a gas anti solvent process comprising
introducing into a particle formation vessel a
supercritical fluid pure or mixed with a modifier; and

a solution comprising said substance and said
stabiliser dissolved in a solvent;

so as said solvent is extracted from the solution
by said supercritical fluid and co-precipitation of the
substance and stabiliser occurs.

Preferably, the solution is introduced into the

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particle formation vessel mixed with a modifier. The
process includes the introduction into a particle
formation vessel of a solution or suspension of the
substance and the stabiliser and a supercritical fluid.
5   In the particle formation vessel, mixing of the
supercritical fluid with the solution and extraction of
the solvent by the supercritical fluid occurs so that
the solutes (substance and stabiliser) co-precipitate as
fine particles. If the solvent is not miscible with the
10   supercritical fluid, the use of a modifier is needed.
The modifier is a compound which is soluble both in the
solvent and in the supercritical fluid.

More preferably, the apparatus in figure 1 is used.
In this case, the solution of substance and stabiliser,
15   the supercritical fluid and the modifier, if needed, are
separately introduced into the particle formation vessel
in co-current flow by the nozzle 27. Such a nozzle
WO02/68107, which is shown in figures 2 and 3, provides
separate inlets for supercritical fluid and solution.
20   In fact, this is a disk with an orifice at its center
and two or more orifices at the same distance from the
center and evenly spaced along a circumference. All the
orifices communicate with the interior of the particle
formation vessel. The solution is introduced into the
25   particle formation vessel through the central orifice,
while the supercritical fluid, pure or with the
modifier, is introduced through the outer orifices.

The modifier and the supercritical fluid are mixed
before the introduction into the particle formation
30   vessel. In another version of the process the modifier
is introduced into the particle formation vessel in part
with the solution and in part with the supercritical
fluid or with the solution only.

The substance is preferably a protein or
35   polypeptide compound of pharmaceutical or diagnostic
interest, soluble in the solvent and in the mixture
solvent/modifier and substantially insoluble in the

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supercritical fluid.

The stabiliser is preferably a pharmaceutical excipient which is able to stabilise the substance in the co-precipitated product. The stabiliser is soluble in the solvent and in the mixture solvent/modifier and substantially insoluble in the supercritical fluid. Preferably, the stabilizer is a sugar and more preferably is trehalose. A mixture of stabilizers may also be employed.

The solvent is preferably selected from water, ethanol, methanol, DMSO, isopropanol, acetone, THF, acetic acid, ethyleneglycol, polyethyleneglycol and N,N-dimethylaniline. Most preferably the solvent is water.

The supercritical fluid is preferably selected from carbon dioxide, ethane, ethylene, propane, sulfur hexafluoride, nitrous oxide, chlorotrifluoromethane, monofluoromethane, xenon and their mixtures, most preferably carbon dioxide.

The modifier is preferably selected from ethanol, methanol, DMSO, isopropanol, acetone, THF, acetic acid, ethyleneglycol, polyethyleneglycol and N,N-dimethylaniline or mixtures thereof. Most preferably the modifier is ethanol. The modifier and solvent must of course be different.

Description of the drawings

Figure 1 shows a schematic flow sheet of the apparatus used to carry out the process according to this invention.

Figures 2 and 3 show the nozzle that is used to carry out the process according to this invention.

Figures 4, 5 and 6 show the particle size distributions of supercritical CO_2 co-precipitated lysozyme/trehalose powders at the w/w ratios 1:10, 1:2 and 1:0 respectively.

Figure 7 shows the thermograms obtained by

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differential scanning calorimetry (DSC) of supercritical
CO_2 co-precipitated lysozyme/trehalose powders vs the
respective pure products.

5   **Detailed description of the invention**

The invention will be described further with
particular reference to the substance being a protein.
It has been found that it is possible to produce stable
10   dry protein/stabilizer micro-particles through the use
of supercritical fluids, using various stabilisers such
as carbohydrates, aminoacids and surfactant polymers.
Surprisingly, it has been found that co-
precipitation using supercritical fluids allows
15   particular intimate interactions between protein and
stabiliser molecules and that for each protein/
stabiliser couple there is an optimal weight ratio. If
the amount of stabiliser exceeds the optimal amount, the
excess does not directly interact with the protein but
20   rather forms particles of pure stabiliser. This
behaviour has been evidenced by Microscopy and by
Differential Scanning Calorimetry (DSC) analysis.
The process for the co-precipitation of a substance
with a stabiliser by a GAS process comprising the use of
25   a supercritical fluid pure or mixed with a modifier and
a solution into a particle formation vessel may be
carried out by the apparatus reported in Figure.
An advantage of the apparatus in figure 1 is
related to the contact between supercritical fluid and
30   solution since this takes place only in the particle
formation vessel. Hence any powder precipitation cannot
occur inside the nozzle and cause blockage. Importantly
the supercritical fluid acts as an anti-solvent but also
promotes conversion of the solution into a fine spray as
35   it enters the particle formation vessel. This widens
the solution/anti-solvent interface and permits a more
rapid mixing of the two phases and hence a rapid protein

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precipitation without any denaturation. In addition, the
enhancement of mass transfer rate between solution and
supercritical fluid allows operation at mild temperature
and pressure conditions which contribute to the
5   avoidance of any possible protein denaturation. The
apparatus of Figure 1 includes a particle formation
vessel 22. This is a standard reaction vessel of an
appropriate volume. The temperature in the vessel is
maintained constant by means of a heating jacket 21.
10   The pressure in the vessel is controlled by means of a
micro metering valve 25.

The temperature and pressure in the particle
formation vessel are measured by means of a thermocouple
29 and a pressure transducer 30.
15   The particles formed are retained by filter 23.
This is a stainless steel basket, whose bottom is made
by a sintered stainless steel disk (0.5 μm). A second
filter 24 (0.5 μm) is put at the vessel outlet.

The supercritical fluid is withdrawn from cylinder
20   3, it is condensed by cooler 4 and pumped by means of
pump 8 to the particle formation vessel through line 34.
Prior to entry into the particle formation vessel, the
supercritical fluid is heated to the desired temperature
by means-of pre-heater 14 and heater 17. The pre-heater
25   14 also acts as pulsation damper. The supercritical
fluid is also filtered by means of filter 15 (0.5 μm).
Temperature and pressure of the supercritical fluid
prior entry into the precipitation vessel are measured
by means of thermocouple 29 and pressure transducer 30,
30   respectively.

The modifier is withdrawn from tank 2, it is pumped
by means of pump 9 to line 34 and it is mixed with the
supercritical fluid prior to entry into the particle
formation vessel. The modifier is also filtered by
35   means of filter 12 (0.5 μm).

Line 34 is equipped with a relief valve 16.

The solution is withdrawn from tank 1, it is pumped

by means of pump 10 to the particle formation vessel
through line 36. The solution is also filtered by means
of filter 13 (0.5 μm).

5   In another version of the process, the modifier may
be introduced into the particle formation vessel in part
with the solution and in part with the supercritical
fluid. The supercritical fluid, pure or mixed with the
modifier, and the solution are fed into the particle
formation vessel by means of the nozzle 27.

10   Downstream the precipitation vessel 22, the mixture
of supercritical fluid, modifier and solvent are
filtered by means of the filter 24 (0.5 μm) to retain
the particles that are eventually not retained by filter
23. The mixture of supercritical fluid, modifier and

15   solvent is depressurised by means of micro metering
valve 25, the supercritical solvent is separated from
the modifier and the solvent in the separator 26, its
flow rate is measured by means of mass flow meter 31 and
it is discharged.

20   The nozzle which is shown in figure 2 and 3 allows
introduction of the solution and the supercritical
fluid, pure or mixed with the modifier, into the
particle formation vessel in co-current flow. The
solution and supercritical fluid velocities at the

25   nozzle outlet are related to the mass flow rate and to
the diameter of orifices. Moreover, it is preferred
that the energy pressure of both solution and
supercritical fluid are converted into kinetic energy
with a minimum energy loss. The nozzle of figures 2 and

30   3 was, in fact, designed for this purpose. The
peculiarity of this nozzle is that the expansion of
solution and supercritical fluid occurs through
orifices. An orifice is characterized by a length to
diameter ratio ranging from 5 to 10. It has the

35   advantage over the capillary of minimizing the pressure
energy loss and of efficiently converting the pressure
energy into kinetic energy. The nozzle has orifices

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with diameters ranging from 0.02 to 0.04 mm and length
ranging from 0.1 to 0.2 mm. Such dimensions allow very
high velocities at the orifice outlet for both solution
and supercritical fluid.

5   The nozzle may be made of stainless steel, or of
other appropriate material.

The nozzle is a disk with an orifice 39 at its
centre and two or more orifices 41 drilled at the same
distance from the centre and evenly spaced along a
10   circumference. The orifices communicate with the
interior of the particle formation vessel. The solution
is introduced into the particle formation vessel through
the central orifice, the supercritical fluid, pure or
with the modifier, is introduced into the particle
15   formation vessel through the outer orifices. The
solution 37 passes through a passage of diameter D3.
Its end has a conical shape 40. At the apex of the
conical end 40 there is a laser drilled orifice 39. The
length L1 of the central orifice is 5 to 10 times its
20   diameter D1. The diameter D1 can be chosen in such a
way to obtain any desired velocity of the solution at
the orifice outlet.

The supercritical fluid 38 passes through passages
of diameter D4. Each passage end has a conical shape
25   42. At the apex of the conical end 42 there is a laser
drilled orifice 41. The length 21 of the orifice is 5
to 10 times its diameter D2. The diameter D2 can be
chosen in such a way to obtain any desired velocity of
the supercritical fluid at the orifice outlet.

30   The ratio between length (L1 or L2) and diameter
(D1 or D2) of the orifices 39 and 41 are chosen so as to
set to a minimum the energy loss and to obtain higher
velocities by converting energy pressure into kinetic
energy.

35   The solution emerges from the central orifice 39 at
high velocity and it is broken in fine droplets coming
in contact with the supercritical fluid. The dispersion

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of the solution liquid jet is enhanced by the
supercritical fluid emerging from orifices 41, provided
that the supercritical fluid velocity is very high, of
the order of magnitude of the velocity of sound at the
5   working temperature and pressure. The effect of the
supercritical fluid in enhancing the dispersion of the
solution liquid jet is crucial and determines the shape,
size and yield of the product.

Orifices can be drilled with diameters down to 0.02
10   mm. The nozzles that have been used for carrying out
the tests have orifices of diameter ranging from 0.02 to
0.04 mm. In another embodiment of the invention, one or
more of the outer orifices are drilled in such a way
that their axes converge on the axis of the central
15   orifice. The angle formed by the axis of the outer
orifices with the axis of the central orifice is between
1 and 30°.

A crucial point in the process for fine dry protein
micro-particle formation is the mixing of the solution
20   with the supercritical fluid: a rapid and intimate
mixing causes precipitation of particles with a small
diameter and enables a high powder yield.

In order to have a good mixing, the solution should
be dispersed into the supercritical fluid in form of
25   small droplets, thus providing high interfacial area for
mass transfer and a short path for the diffusion of
supercritical fluid in the solution droplets and thereby
preventing the growth of solute particles. Moreover a
high ratio between flow rate of supercritical fluid and
30   flow rate of solution causes a large excess of the
supercritical fluid to solution at the moment of their
contact, enhancing the driving force for mass transfer
of the supercritical fluid into the solution and of
solvent into supercritical fluid.
35   When the solvent solubility in the supercritical
fluid is low, the use of a modifier allows a better
mixing between solution and supercritical fluid.

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When a modifier is used, the ratio of modifier flow
rate and of solution flow rate has to be chosen so that
a high increase in solubility of solvent in the
supercritical fluid is obtained. The modifier can be
5   introduced with the supercritical fluid or with the
solution or in part with the supercritical fluid and in
part with the solution. The way of introduction of the
modifier greatly influences the extraction of the
solvent and the structure of particles that are formed.

10   For the precipitation of powders from aqueous
solution using carbon dioxide as supercritical solvent
and ethanol as modifier the ratio between supercritical
fluid flow rate and the modifier flow rate is preferably
within the range 4-8, more preferably 7, while the ratio
15   between modifier flow rate and the solution flow rate is
preferably within the range 15-25 and more preferably
20.

As pointed out above, it is necessary to have a
good dispersion of the solution into the supercritical
20   fluid in order to obtain very small droplets of
solution.

The size of the formed solution droplets is
determined by the fluidodynamic conditions in the mixing
zone and by the physical properties of solution and
25   supercritical solvent, such as viscosity, surface
tension, density. These properties are greatly
influenced by the temperature and pressure of the
supercritical fluid.

The supercritical fluid inlets are positioned
30   around the solution inlet and at a very short distance
therefrom (about 3 mm): this configuration allows the
solution to be energized by the supercritical fluid thus
enhancing the dispersion of the solution into very fine
droplets, providing high interfacial surface are between
35   the two phases and fast extraction of solvent into
supercritical fluid. These phenomena are particularly
efficient when the supercritical fluid velocity at the

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orifice outlet reaches or is greater than the speed of
sound causing a Mach disc formation and solution
dispersion as very fine droplets (Matson D.W., Fulton J.
L., Petersen R. C., Smith R. D., "*Rapid expansion of*
5 *supercritical fluid solutions: solute formation of
powders, thin films, and fibers*" Ind. Eng. Chem. Res.,
1987 26, 2298-2306). The speed of sound in a fluid is
strongly dependent on pressure and temperature: the
minimum value of speed of sound for carbon dioxide in
10 the supercritical region is 208 m/s at 8 MPa and 40°C.
To take advantage of the above mentioned phenomena it is
convenient to work around the value of speed of sound
for carbon dioxide in the supercritical region, e.g. 208
m/s at 8 MPa and 40°C.

15   For the production of fine powders from aqueous
solutions with the GAS process using carbon dioxide as
supercritical solvent and ethanol as modifier, it was
found that optimal operative conditions are 8-12 Mpa and
35-50°C. In the experimental apparatus used for
20 carrying out the experimental tests, the supercritical
fluid mass flow rate was 30 g/min, the solution flow
rate 0.2 g/min, and the modifier mass flow rate 4 g/min,
having set the ratio of supercritical fluid to modifier
mass flow rate at 7 and the ratio of modifier to
25 solution mass flow rate at 20 and supercritical fluid
velocity at the nozzle outlet at about 300 m/s. Using
this apparatus we carried out the process to produce
stable dry micro-particles of a substance and a
stabiliser by GAS co-precipitation. Proteins such as
30 Alkaline Phosphatase and Lysozyme were used as the
substance and Trehalose as stabiliser. Co-precipitated
powders at different protein/stabiliser ratio were
produced. The yield of the collected powder was 90%.
The retained enzymatic activity after the process was
35 found to be within 95% and 100%, compared to the
unprocessed commercial reagent. The particle size
distributions of these powders showed that more than 90%

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of the particles have an equivalent diameter less then
10 μm with a narrow size distribution. Furthermore, the
physico-chemical characterisation showed that co-
precipitation allows intimate interactions between
5   protein and stabiliser molecules and for each protein/
stabiliser couple there is an optimal weight/weight
ratio. Finally, stability studies showed that the
Alkaline phosphatase/trehalose co-precipitated particles
were more stable than the equivalent freeze-dried
10   product.

Experimental procedure

The supercritical fluid is fed to the precipitation
15   vessel by means of pump 8, which is used to set the
supercritical fluid flow rate. The temperature of the
supercritical fluid in line 35 is set by means of heater
17 to a higher value than the temperature inside the
particle formation vessel, to take into account the
20   temperature lowering due to the expansion through the
nozzle orifices. The modifier is then added at a
predetermined flow rate to the supercritical fluid by
means of pump 9. The solution of protein and stabiliser
is pumped by means of pump 10 into the particle
25   formation vessel when steady state conditions are
attained.
        After that a certain amount of solution is fed into
the particle formation vessel, pumps 9 and 10 are
stopped and only the supercritical fluid is fed to the
30   particle formation vessel as long as the precipitated
powder is free of solvent and modifier.
        The particle formation vessel is depressurised, the
powder is recovered and sealed in 10 mL vials under dry
nitrogen.
35   The co-precipitated proteins stability was tested
by storing vials at the following conditions:
25°C - 60% RH; 30°C - 65% RH; 40°C - 75% RH. Each

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sample was analysed for biological activity at t= 0, 1,
2, 3 and 6 months. As comparison, a parallel study was
conducted on protein precipitated by supercritical
fluids as is, on analogous freeze-dried products and on
5   the unprocessed commercial product, all stored under dry
nitrogen.

Example 1
Preparation of alkaline phosphatase (ALP)/trehalose co-
10 **precipitate particles**

In this example, the method of the invention is
used to co-precipitate mixtures of alkaline phosphatase
(ALP) and trehalose.
15   Solutions containing ALP (SIGMA Chemicals) at
concentration 0.2% w/w and trehalose (SIGMA Chemicals)
at concentration within the range 0-2% w/w in deionized
water were used.
The ALP/Trehalose ratios of the obtained powders
20 were as follow: 1:10, 1:2 and 1:0. Carbon dioxide as
supercritical fluid and ethanol as modifier were used.
The solution was fed into the particle formation
vessel 22 by means of pump 10 at a flow rate of 0.2
g/min. Supercritical carbon dioxide was fed by means of
25 pump 8 at a flow rate of 30 g/min, ethanol was fed by
means of pump 9 to line 34 at a flow rate of 4 g/min and
it was mixed with supercritical carbon dioxide prior to
entry into the particle formation vessel.
The supercritical fluid was injected into the
30 particle formation vessel through the four external
orifices of the nozzle, each with a diameter of 0.04 mm.
The solution was injected into the particle formation
vessel through the central orifice of the nozzle, having
a diameter of 0.04 mm. The length of all orifices was
35 0.2 mm.
Temperature and pressure inside the particle
formation vessel were maintained at a constant 40°C, by

means of the heating jacket 21, and 100±1 bar by means
of the micro metering regulation valve 25, respectively.
Precipitated particles were collected on the filter 23
at the bottom of particle formation vessel, while
5   supercritical fluid, modifier and water were collected
into the cylinder 26 at atmospheric pressure.

The process was conducted as long as a sufficient
amount of powder was obtained. After the solution and
modifier feeds were stopped and only pure carbon dioxide
10   was fed into particles formation vessel in order to
extract any trace of solvent and modifier from the
precipitated powders. Typically, the particle formation
vessel was washed with two volumes of carbon dioxide in
order to obtain dry powders.

15   After depressurisation, the particle formation
vessel was opened and the powders were recovered and
stored in 10 mL vials under dry nitrogen.

The yield of the collected powder was 90%.

The residual enzymatic activity of ALP was within
20   95% and 100%, compared to the unprocessed commercial
reagent. The powder optical microscopy analysis shows
that at high trehalose content as for ALP/trehalose
ratio 1:10 the powder is formed by two different
population of particles: one, which is the largely
25   frequent, is formed by needle shaped particles, while
the other by round shaped particles. The needle shaped
particles are quite similar to those obtained by
trehalose as is precipitation by supercritical CO_2. The
lower trehalose content powders show the round shaped
30   particles population only. Thus, the trehalose can be
co-precipitated with ALP by supercritical CO_2 to give one
kind of particles only at the lower trehalose contents
(protein/trehalose ratios 1:2). Similar behaviour was
found for lysozyme/trehalose co-precipitates (see
35   example 2). Analogous products were prepared by freeze-
drying. In this case the found residual enzymatic
activity of ALP was within 95% and 104%, compared to the

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unprocessed commercial reagent.

Similar vials containing unprocessed commercial ALP
or analogous freeze-dried products, all under dry
nitrogen, were prepared.

5

Stability Study

Several vials of each category were placed at each
of the following conditions: 25°C - 60% RH; 30°C - 65%
10   RH; 40°C - 75% RH for 6 months. At t = 0, 1, 2, 3, 6
months, the contents of the vials were assayed for ALP
activity. The stability studies results are summarised
in Table 1.

.Pure ALP precipitated by supercritical CO_2 (sample
15   F6) shows a decay of enzymatic activity at all the
conditions. The residual activity after 6 months at
40°C - 75% RH (most extreme conditions), is 57% of the
t=0 value.

On the contrary, no significant loss of activity at
20   all conditions up to 6 months was found for
ALP/trehalose co-precipitated by supercritical CO_2 at
ratio 1:10 (sample FT8).

At 40°C - 75% RH, pure freeze-dried ALP (sample F8)
and SIGMA commercial product show similar decays and
25   only 43% and 42% of the initial enzymatic activity was
retained after 6 months. At the other conditions,
instead, the SIGMA product shows a slower activity loss
than the pure freeze-dried ALP. In fact after 6 months
the following residual enzymatic activity were detected:
30   95% vs 83% at 25°C - 60% RH, and 86% vs 76% at 30°C -
65% RH.

Finally, the freeze dried powder having the
ALP/trehalose ratio of 1:10 (sample FT10) showed an
initial rapid loss of activity, then a slower one up to
35   six months which seams to be independent to the storage
conditions. In fact the retained enzymatic activities
at 250°C - 60% RH, 30°C - 65% RH and 40°C - 75% are 90%,

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88% and 90% respectively of the initial value.

Example 2

Preparation of lysozyme/trehalose co-precipitate
5   **particles**

In this example, the method of the invention is used to prepare co-precipitate powders using lysozyme and trehalose.

Solutions containing lysozyme (SIGMA Chemicals) at
10   concentration within 0.2 - 1% w/w and trehalose (SIGMA Chemicals) at concentration within the range 0 - 2% w/w in deionized water were used. The lysozyme /trehalose ratios of the obtained powders were as follows: 1:10, 1:5, 1:2, 1:1, 2:1, 4:1 and 1:0 (Table 2).

15   Carbon dioxide was used as supercritical fluid and ethanol as modifier.

The aqueous solution containing the enzyme and the stabiliser was fed into the particle formation vessel 22 by means of pump 10 at a flow rate of 0.2 g/min.
20   Supercritical carbon dioxide was fed by means of pump 8 at a flow rate of 30 g/min, ethanol was fed by means of pump 9 to line 34 at a flow rate of 4 g/min and it was mixed with supercritical carbon dioxide before entering into the particle formation vessel.

25   The supercritical fluid was injected into the particle formation vessel through the four external orifices of the nozzle, each with a diameter of 0.04 mm. The solution was injected into the particle formation vessel through the central orifice of the nozzle, having
30   a diameter of 0.04 mm. Length of all orifices is 0.2 mm.

Temperature and pressure inside the particle formation vessel were maintained at a constant 40°C, by means of the heating jacket 21, and 100±1 bar by means
35   of the micro metering regulation valve 25, respectively.

Precipitated particles were collected on the filter 23 at the bottom of particle formation vessel, while

supercritical fluid, modifier, water and solute
eventually not precipitated were collected in the
cylinder 26 at atmospheric pressure.

5   After that, a certain amount of solute was fed into
the particle formation vessel, pumps 9 and 10 were
stopped and only supercritical fluid was fed into the
particle formation vessel in order to dry the
precipitated powders: typically, it requires about two
times the volume of the particles formation vessel to
10   obtain dry powders.

At this point, the particle formation vessel was
depressurised, opened and the powders collected.

The yield of recovered powders was 90%.

The found residual enzymatic activity of lysozyme
15   was within 96% and 100%, compared to the unprocessed
commercial reagent.

Table 2 reports for each sample the lysozyme/
trehalose ratio, the retained enzymatic activity, the
protein content which is related to homogeneity of
20   precipitation, the number of particles populations and
the particle sizes. As can be noted, for all the
samples, both enzymatic activity and protein content are
very close to theoretical values. Thus the experimental
conditions we used allowed a similar precipitation for
25   both protein and sugar and guaranteed an almost complete
biological activity recovery.

The particle size distributions of powders
calculated by image analysis of SEM micrographs showed
that for all the powders obtained by supercritical CO_2
30   precipitation, more than 90% of the particles have an
equivalent diameter less then 10 μm with a narrow size
distribution. Figures 4, 5, 6 show the particle size
distributions of supercritical CO_2 co-precipitated with
lysozyme/trehalose ratios 1:10, 1:2 and 1:0
35   respectively. The other co-precipitates yielded similar
distributions. Moreover, the powders observation by
optical microscopy analysis showed that at high

trehalose contents as for both lysozyme/trehalose ratios
1:10 and 1:5, the powders were composed of two particle
populations: the one, which largely was the most
frequent, was formed by needle shaped particles, the
5   other was formed by round shaped particles. The needle
shaped particles were quite similar to those obtained by
trehalose as precipitated by supercritical CO_2. On the
contrary, at the lower trehalose content (lysozyme/
trehalose ratio 1:2) the powders showed the round shaped
10   particles population only. Thus lysozyme can be co-
precipitated with trehalose by supercritical CO_2 to form
only one kind of particle at the lower trehalose content
(higher protein/trehalose ratios). Hence an optimal
value for protein/trehalose ratio which guarantees the
15   best interaction between the two kind of molecules
exists. This behaviour has been confirmed by DSC
analysis. Figure 7 shows DSC thermograms of various co-
precipitated lysozyme powders. As reference, pure
precipitated lysozyme and trehalose are also reported.
20   As can be noted, at higher trehalose content (ratio
1:5), samples contain amorphous trehalose which recovers
the crystalline abit (exothermic peak at 197°C) and then
melts at 214°C in the same way of precipitated trehalose
itself. The thermal behaviour of lower content
25   trehalose samples is quite different. The samples with
ratios 1:2 to 4:1 show thermograms similar to the one of
lysozyme as is. The most relevant difference is the
shift towards lower temperatures of the characteristic
transition of lysozyme at T=204°C. The higher the
30   trehalose content the lower the temperature of
transition. Thus, we have strong evidence that co-
precipitation by supercritical fluids allows an intimate
interaction between protein and trehalose. In fact up
to a defined amount of sugar (1:2 ratio) we obtained a
35   homogeneous solid phase. This ratio is able to provide
the best protein/sugar interaction and the best long
term stability of protein.

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TABLE 1

Sample/ drying method	ALP/ Trehalose Ratio	Storage condition	Enz. Activity at 1 month (% of t=0)	Enz. Activity at 2 months (% of t=0)	Enz. Activity at 3 months (% of t=0)	Enz. Activity at 6 months (% of t=0)
ALP Sigma Freeze-drying	1:0	-20°C	—	—	—	96
		25°C/60%RH	101	100	101	95
		30°C/70%RH	103	103	94	86
		40°C/75%RH	94	85	75	42
ALP F6 SCF	1:0	-20°C	—	—	—	—
		25°C/60%RH	91	72	69	67
		30°C/70%RH	101	71	59	59
		40°C/75%RH	64	69	58	57
ALP F8 Freeze-drying	1:0	-20°C	—	—	—	—
		25°C/60%RH	88	89	89	83
		30°C/70%RH	83	87	87	76
		40°C/75%RH	75	75	62	43
ALP FT8 SCF	1:10	-20°C	—	—	—	101
		25°C/60%RH	111	99	113	99
		30°C/70%RH	104	97	102	99
		40°C/75%RH	113	98	101	99
ALP FT10 Freeze-drying	1:10	-20°C	—	—	—	95
		25°C/60%RH	95	95	93	90
		30°C/70%RH	94	96	92	88
		40°C/75%RH	94	93	92	90

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TABLE 2

SAMPLE	Lysozyme / Trehalose Ratio	Enz. Activity (mg Enz.Img Prot.)	Protein content (% on nominal)	No. of particles populations	Particles size (% < 10 jim)
L3	1:0	0.96	102.6	1	99
LT2	1:10	1.04	104.3	2	——
LT3	1:1	0.96	100.8	1	98
LT6	1:5	0.96	104.0	2	92
LT8	4:1	1.01	103.2	1	97
LT9	1:2	0.98	104.0	1	93
LT10	2:1	0.97	103.9	1	97

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CLAIMS

1.   A process for the co-precipitation of a substance
with a stabiliser therefor, by a gas anti solvent
process comprising introducing into a particle formation
vessel a supercritical fluid pure or mixed with a
modifier; and
     a solution comprising said substance and said
stabiliser dissolved in a solvent;
     so as said solvent is extracted from the solution
by said supercritical fluid and co-precipitation of the
substance and stabiliser occurs.

2.   A process as claimed in claim 1 wherein the
substance is a protein or polypeptide.

3.   A process as claimed in claim 2 wherein the
substance is a protein.

4.   A process according to claim 1 to 3 wherein said
solution and said supercritical fluid are introduced
into said particle formation vessel via separate inlet
nozzles.

5.   A process according to claim 4 wherein said
supercritical fluid is introduced into said particle
formation vessel via a plurality of inlet nozzles.

6.   A process as claimed in claim 5 wherein said
nozzles are present on a disk, the solution inlet nozzle
being in the centre of said disk surrounded by a
plurality of supercritical fluid inlet nozzles evenly
spaced along the circumference thereof.

7.   A process according to claim 1 to 6, wherein the
solution is introduced into the particle formation
vessel mixed with a modifier.

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8.   A process according to claim 1 to 7 wherein the supercritical fluid is introduced into the particle formation vessel mixed with a modifier.

9.   A process according to any of claims 1 to 8, wherein said supercritical fluid is selected from carbon dioxide, ethane, ethylene, propane, sulfur hexafluoride, nitrous oxide, chlorotrifluoromethane, monofluoromethane, xenon and mixtures thereof.

10.   A process according to any of claims 1 to 9, wherein said solvent is selected from water, ethanol, methanol, DMSO, isopropanol, acetone, THF, acetic acid, ethyleneglycol, polyethyleneglycol and N,N-dimethylaniline and mixtures thereof.

11.   A process according to any of claims 1 to 10, wherein said modifier, which is different from said solvent is selected from water, ethanol, methanol, DMSO, isopropanol, acetone, THF, acetic acid, ethyleneglycol, polyethyleneglycol and N,N-dimethylaniline and mixtures thereof.

12.   A process according to any of claims 1 to 11, wherein the supercritical fluid is carbon dioxide, the solvent is water and the modifier is ethanol.

13.   A process according to claims 1 to 12 wherein the stabiliser is a sugar.

14.   A process according to claim 13, wherein the sugar is trehalose.

15.   A process according to any one of claims 1 to 14 wherein the ratio of substance to stabiliser in the solution is 1:1 to 1:10.

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16.   A process according to claim 15 wherein the ratio of substance to stabiliser in the solution is 1:2.

17.   A process as claimed in any one of claims 1 to 16 wherein the supercritical fluid enters the particle formation vessel at the speed of sound in the fluid or greater.

18.   A process as claimed in any one of claims 1 to 17 where a modifier is used and the ratio between supercritical fluid flow rate and modifier flow rate is within the range 4:1 to 8:1.

19.   A process as claimed in any one of claims 1 to 18 where a modifier is used and the ratio between modifier flow rate and solution flow rate is within the range 15:1 to 25:1.

20.   A coprecipitate of substance and stabiliser obtained by a process as claimed in any one of claims 1 to 19.
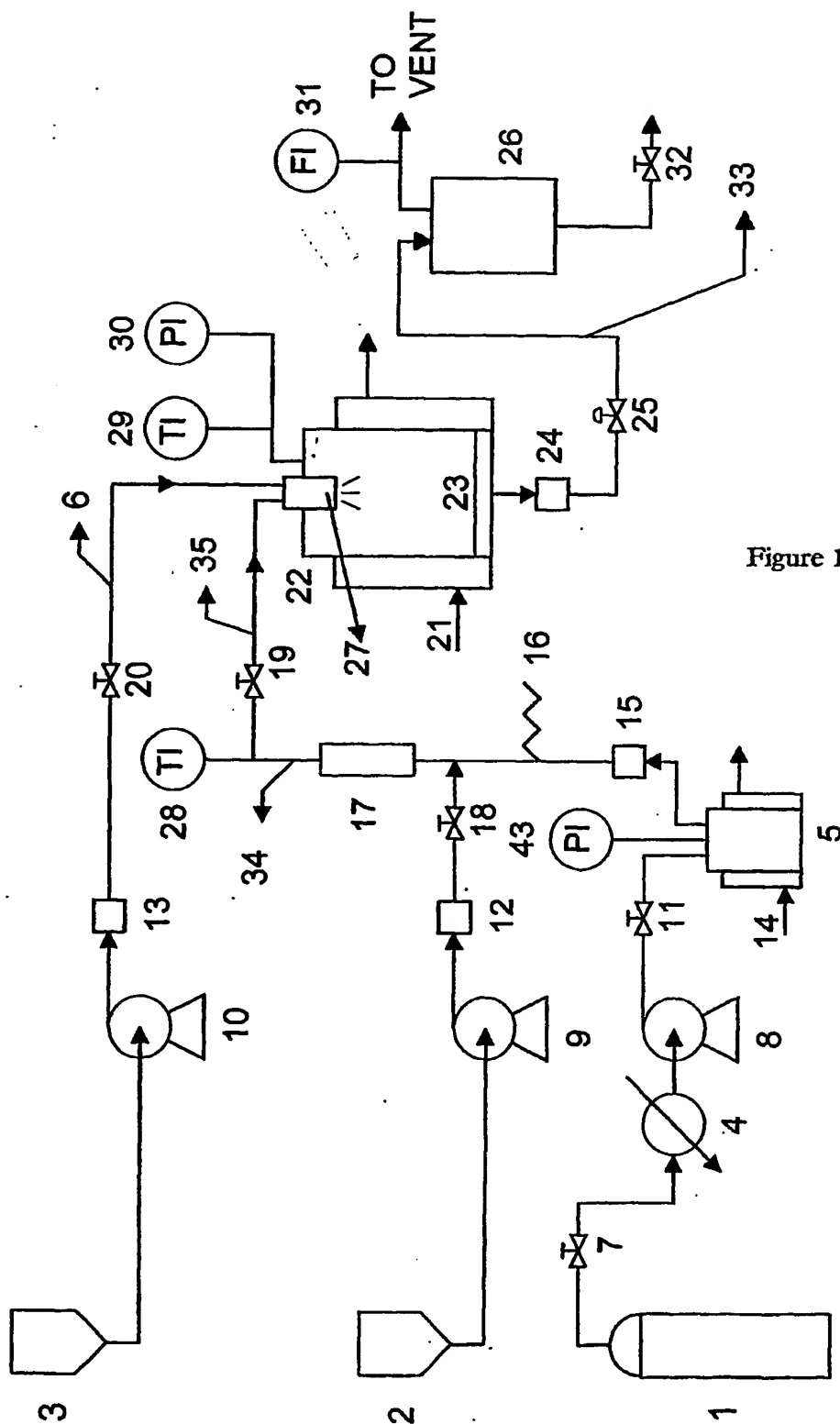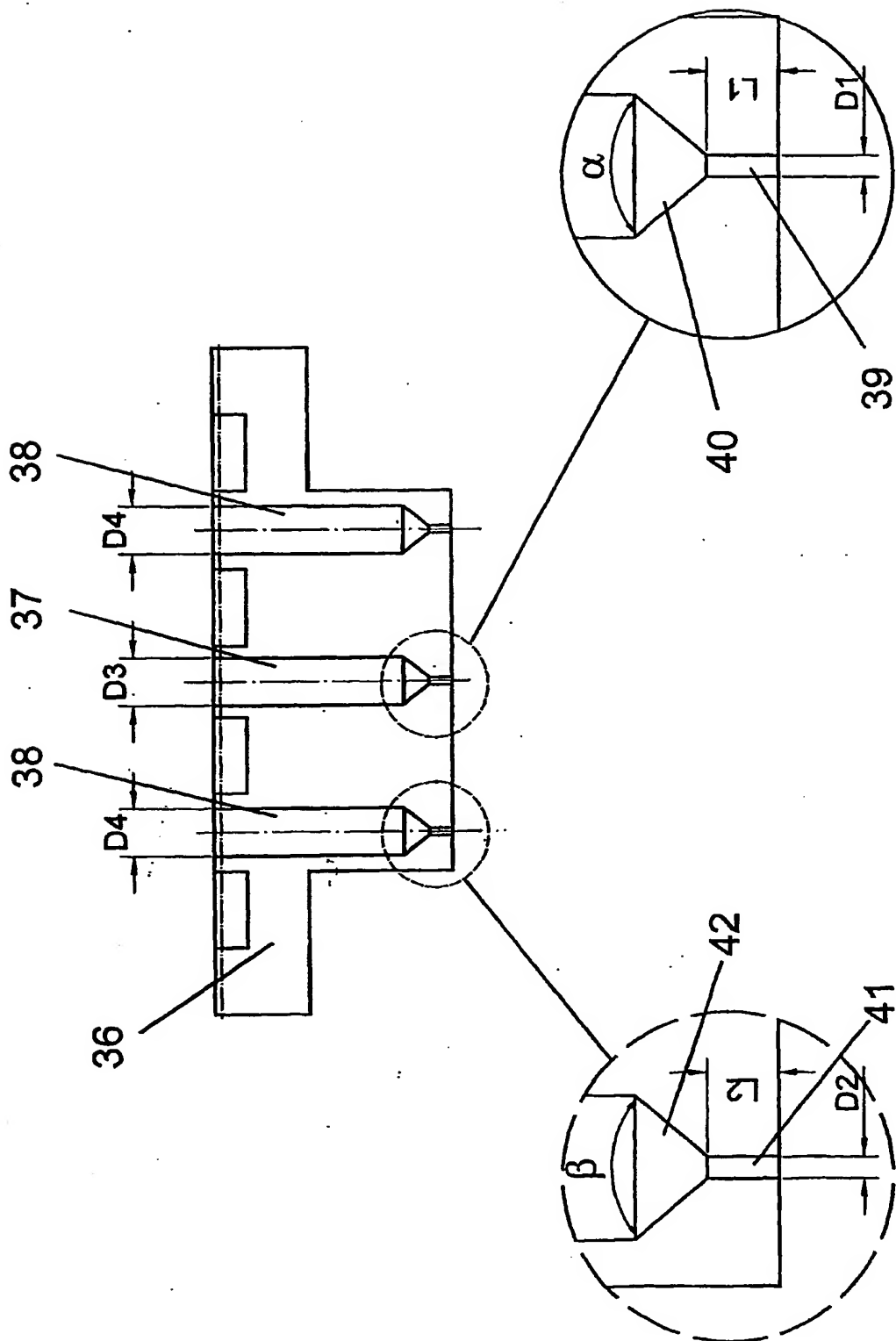
Figure 1

Fig. 2.

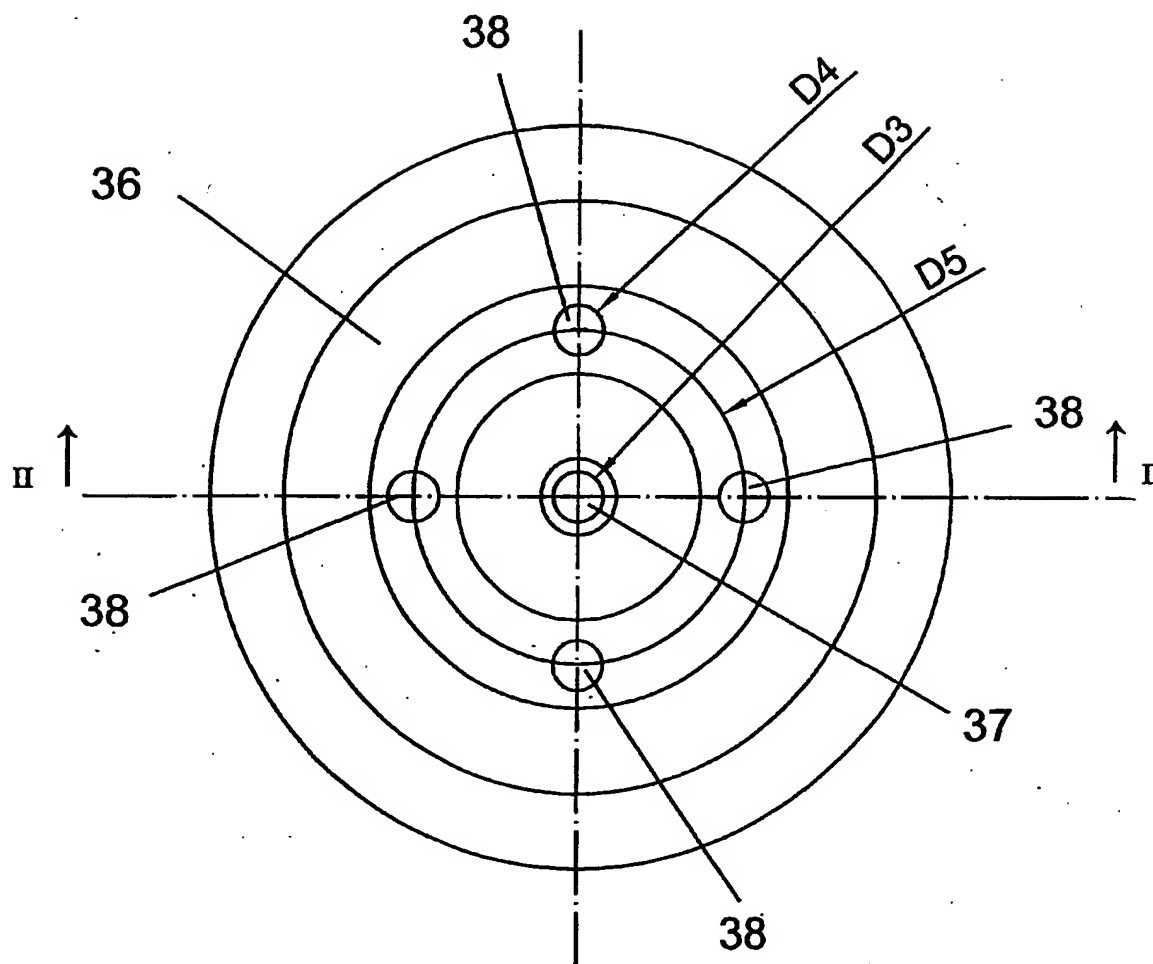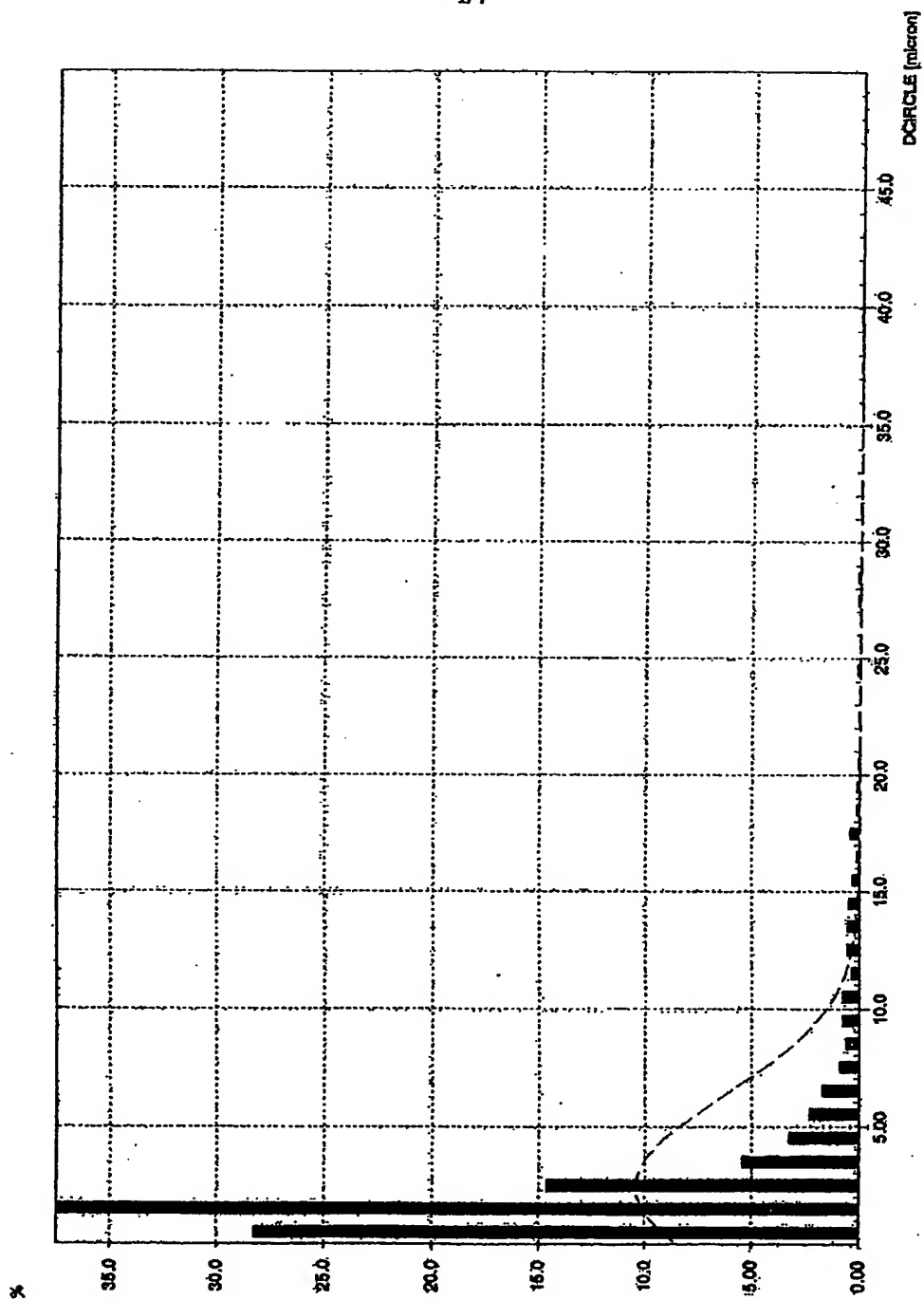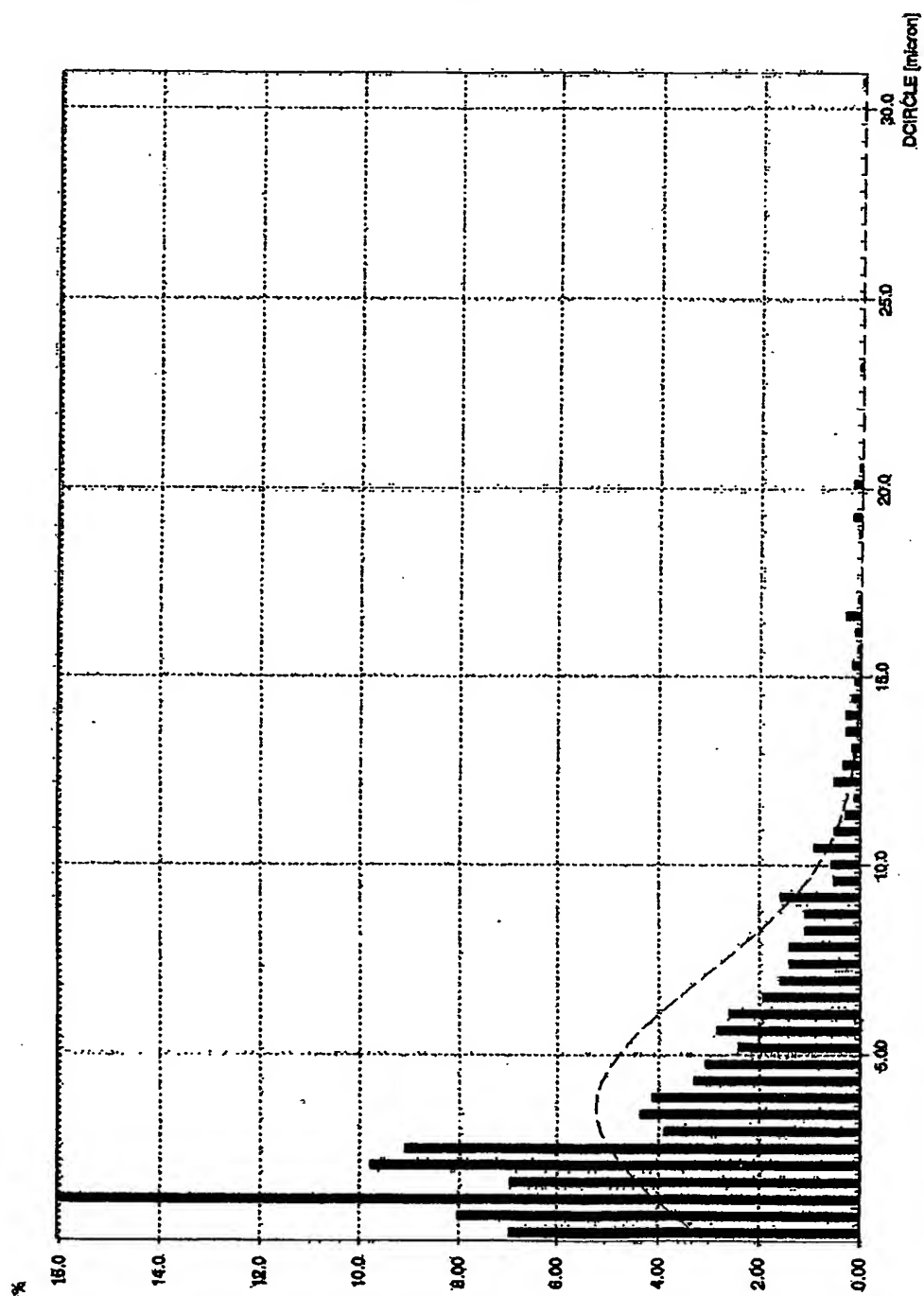Figure 3
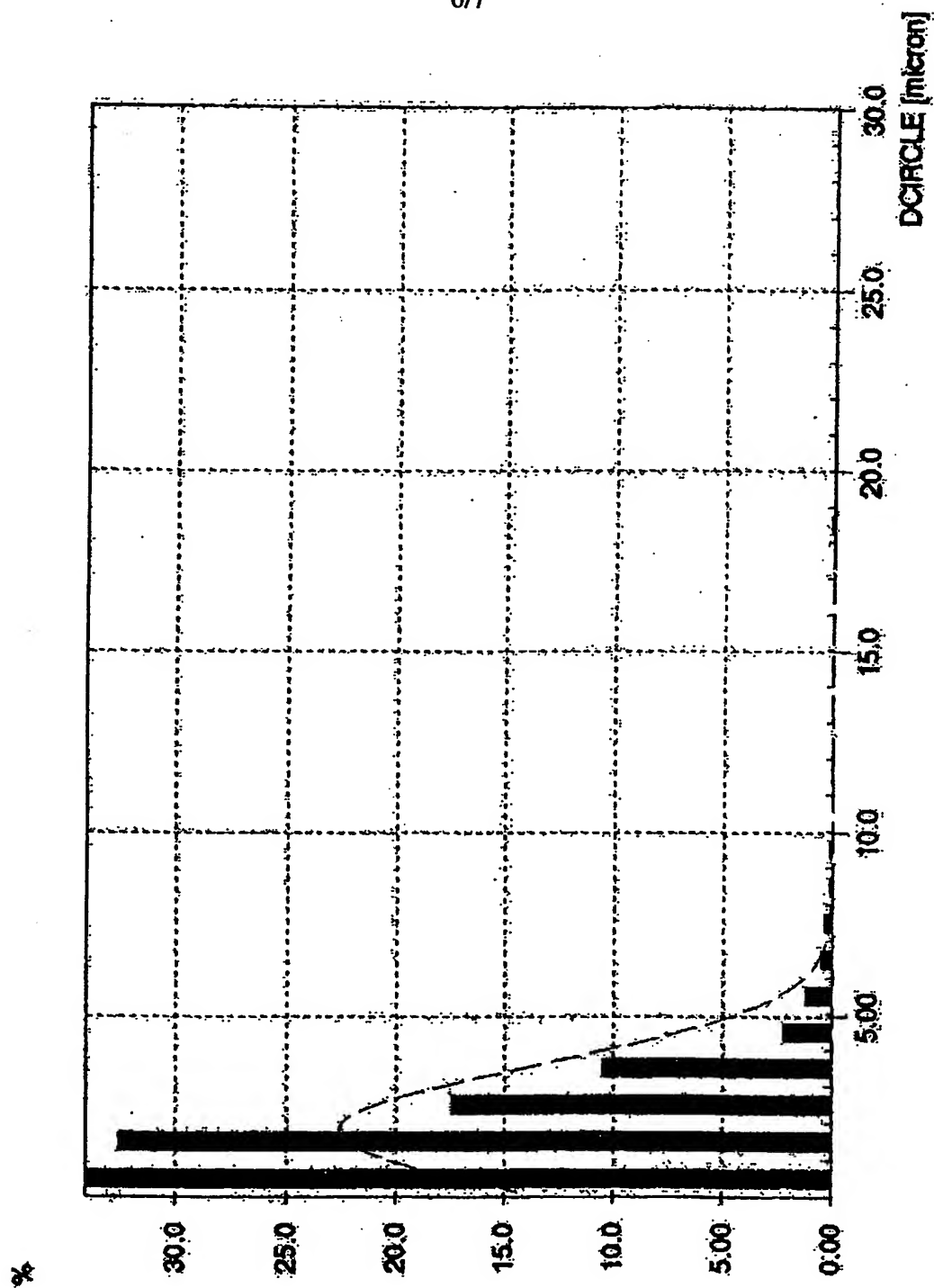
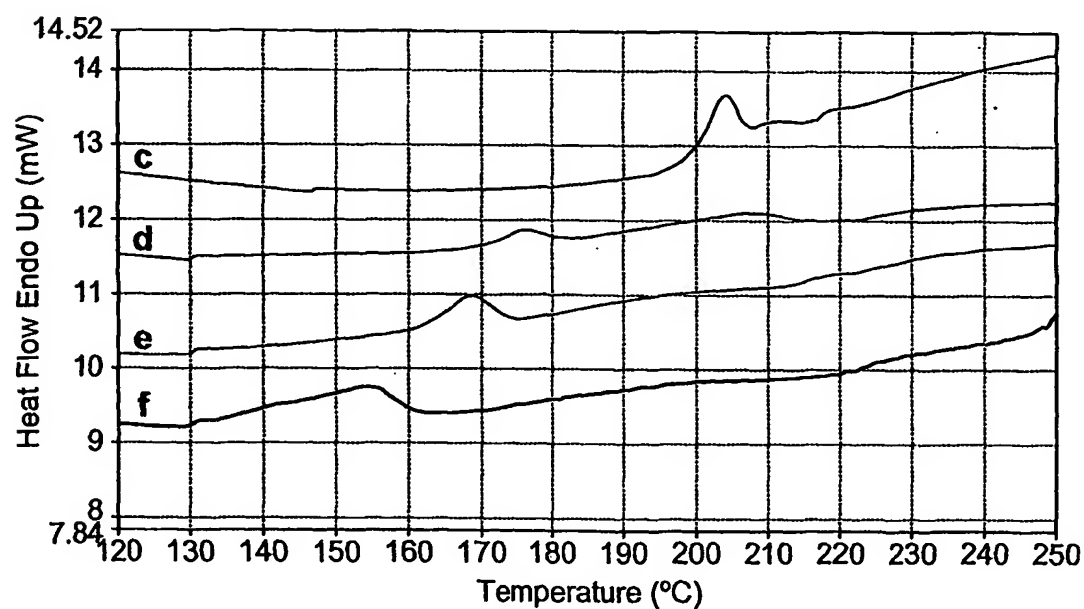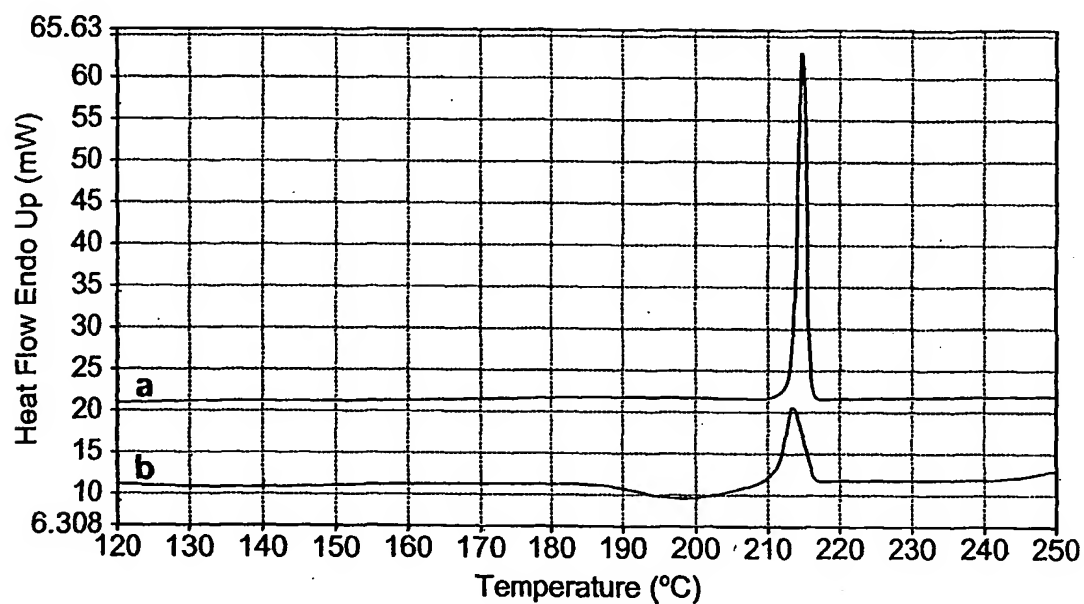Figure 4.

Figure 5.

6/7

Figure 6.

FIG. 7

Thermal analysis of supercritical CO2 co-precipitated lysozyme/trehalose powders.
a: pure trehalose; **b**: lysozyme/trehalose 1:5 ratio; **c**: pure lysozyme; **d**: lysozyme/
trehalose 4:1 ratio; **e**: lysozyme/trehalose 2:1 ratio; **f**: lysozyme/trehalose 1:2 ratio.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C07K1/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C07K   B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 770 559 A (RANDOLPH THEODORE W ET AL) 23 June 1998 (1998-06-23) column 8, line 56 – line 61 column 10, line 19 – line 23 examples 19-32	1-20
X	JUNG J ET AL: "Particle design using supercritical fluids: Literature and patent survey" JOURNAL OF SUPERCRITICAL FLUIDS, PRA PRESS, US, vol. 20, no. 3, August 2001 (2001-08), pages 179-219, XP004247117 ISSN: 0896-8446 page 187 –page 201; figures 2-4; table 2	1-20

-/—

| [X] | Further documents are listed in the continuation of box C. | | [X] | Patent family members are listed in annex. |

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Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2003	18/02/2003

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	examples 1-4	20
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